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# Structure of the Inverted Terminal Repetition of Adenovirus Type 2 DNA[1]

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Several secondary structure features involving the ends of single strands of adenovirus type 2 DNA have been studied by electron microscopy by both the gene 32-ethidium bromide technique and a modification of the standard formamide-cytochrome *c* technique. A duplex stem of length 115 ± 10 nucleotide pairs due to pairing between the two members of the inverted terminal repetition is observed in the single-stranded circles that form upon annealing single-stranded linear molecules. This duplex stem is shown to lie at the ends of the DNA by using several reference markers: (i) a newly discovered secondary structure feature (a loop of length ca. 500 nucleotides with a 20-nucleotide pair duplex stem) that maps 73% of the full length from the left end of the molecule and (ii) a duplex region due to a hybridized restriction fragment. There is also some secondary structure within each end of linear single strands. There is some variation in the morphology of the end structures, and we propose that these involve base pairing, as in a tRNA clover leaf, rather than an exact single hairpin-type inverted repeat. These observations are consistent with the hypothesis that there is a foldback structure at the 3′ ends of the DNA that functions as a primer for the initiation of replication.

Adenovirus type 2 (Ad2) DNA contains an inverted terminal repetition that enables the single-stranded linear DNA to circularize under suitable incubation conditions (7, 20). An estimate of the length of the inverted terminal repetition is based upon the observations that the restriction endonucleases *Hha*I and *Hph*I cut fragments of an identical length of about 70 and 100 nucleotide pairs (ntp), respectively, from the two ends of the duplex DNA, whereas the enzymes *Hae*II and *Hpa*II release fragments of length about 140 ntp from the left end but release much larger fragments from the right end (16). These results suggest that the length of the inverted repetition lies between 100 and 140 ntp. Earlier estimates, by less reliable methods, gave values between 200 and 500 ntp (7, 29). The short length of 100 to 140 ntp explains why it has not yet been possible to identify the duplex stem and measure its length by electron microscopy (EM) on the long single-stranded circles (contour length, 34 kilobases [kb]) mounted for EM by the formamide-cytochrome *c* method.

Similar inverted terminal repetitions were reported for the DNAs of adenovirus serotypes 1, 3, 7, 18, and 31 (7). In the case of adeno-associated virus (2, 9) the terminal duplex formed by circularization was visualized by EM, and its length was estimated to lie between 40 and 100 ntp (2). Electron microscope observations are technically easier for this DNA because of its shorter length (5 kb).

Robinson et al. (17) showed that there is a protein tightly associated with the ends of Ad2 DNA. Recently, it was shown by R. J. Roberts that this protein is covalently bound at the 5′ ends to both DNA strands (personal communication). The biological function of this protein, like that of the terminal repetition, is unknown; however, a model has been proposed in which the protein plays a role in the initiation of DNA replication (Roberts, personal communication). The model requires that a foldback (or palindromic) sequence be present at the end of the linear Ad2 DNA to prime replication in a manner similar to that suggested previously by Cavalier-Smith (4). The first step in this model requires strand separation followed by foldback of the 3′ strand to set up a template-primer situation as shown in Fig. 1. Some experimental evidence is available implicating structures of this sort during the replication of adeno-associated virus (18) and some other nondefective parvoviruses (3). Furthermore, there is ev-

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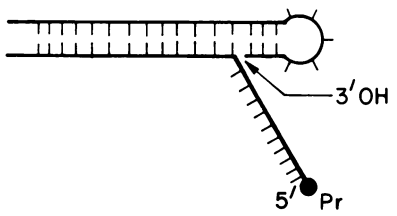



FIG. 1. *Sketch of the fold-back structure proposed by Roberts (in press) for the initiation of replication of Ad2 DNA. Pr represents the protein covalently attached to the 5' end.*

idence of inverted repeats close to the ends of Ad2 DNA (1).

Therefore, we undertook a further electron microscope study of the structure of the ends of Ad2 DNA. For this purpose, we used several different electron microscope mounting methods. The newly developed gene 32-ethidium bromide (EtdBr) technique, in which T4 gene 32 protein is selectively bound to single-stranded DNA, gives particularly good discrimination between duplex and single-stranded regions on a DNA (21). The average interbase distances of both gene 32 protein-coated single-strand DNA and EtdBr intercalated double-strand DNA are between 0.49 and 0.52 nm. These spacings are considerably longer than the values of 0.31 and 0.34 nm per nucleotide or nucleotide pair for single- and double-stranded DNA, respectively, in conventional formamide-cytochrome *c* spreading. The extended lengths make the method particularly suitable for the study of a DNA with short interspersed double-stranded and single-stranded regions. The EtdBr technique is useful for high-resolution visualization of duplex DNA (8, 10). The standard cytochrome *c*-formamide mounting method (5) was also used; spreadings from lower percent formamide solvents were used to increase the frequency with which certain secondary structure features could be observed (22).

## MATERIALS AND METHODS

Ad2 DNA was prepared as described previously (14) and digested with *Escherichia coli* exonuclease III (Exo III) for 10 min as described (16). The restriction endonuclease fragments, *Hpa*I-E, *Eco*RI-E and *Hin*dIII-B, were isolated from complete digests by agarose gel electrophoresis, using the hydroxyapatite elution method (19). $\phi 80d_3ilv$ DNA was prepared as described previously (25). T7 DNA was a gift from P. Serwer.

The technique for conventional 50% formamide-cytochrome *c* spreading of DNA was described previously (5). For spreading under less denaturing conditions, the spreading solution contained 38% formamide, 0.08 M Tris (pH 8.4), 8 mM EDTA, 80 μg of cytochrome *c* per ml, and 0.1 to 0.5 μg of DNA per ml. The hypophase was 8% formamide-10 mM Tris-1 mM EDTA (22). The gene 32-EtdBr technique was described previously (21). The EtdBr technique was according to Koller et al. (10) as modified by Kasamatsu and Wu (8).

In the previous restriction-endonuclease-mapping study, all length measurements were relative to full length Ad2 DNA. We found a length ratio of 6.53 ± 0.34 between duplex Ad2 DNA and open circular φX174 RF in regular 50% formamide-cytochrome *c* spreads. We took the length of φX174 as 5.2 kb; Ad2 DNA then has a length of 34.0 ± 1.8 kb.

In presenting results, we reported lengths either in nucleotides, kilobases, or in Ad2 units, with 1 U equal to 1% of the length of Ad2 DNA, i.e., 0.34 kb. The origin of the coordinate system on Ad2 DNA was left end, as assigned previously (16).

Circular single strands of Ad2 DNA were prepared as follows: 50 μl of formamide was mixed with 0.5 μl of 1 M Tris, 0.1 M EDTA (pH 8.4), and 2 μl of duplex linear Ad2 DNA (100 μg/ml in 0.01 M Tris-1 mM EDTA). The mixture was incubated at 37°C for 5 min to denature the duplex DNA and then 45 μl of cold water and 4.5 μl of 1 M Tris-0.1 M of EDTA was added. This renaturation solution was incubated at room temperature for 1 h. To hybridize denatured restriction fragments to single strands of Ad2 DNA, 1 μl of Ad2 DNA and 1 μl of restriction fragment *Eco*RI-E or *Hpa*I-E (all at 100 μg of DNA per ml) were mixed with 50 μl of formamide, 0.5 μl of 1 M Tris, and 0.1 M EDTA (pH 8.4), and then denatured at 37°C for 5 min. After addition of 45 μl of water and 4.5 μl of 1 M Tris-0.1 M EDTA (pH 8.4), the mixture was incubated at room temperature for 30 min; then a second solution (0.2 μg) of denatured restriction fragment in 50 μl of 50% formamide-0.1 M Tris-0.01 M EDTA was added. The mixture was kept at room temperature for an additional 30 min and then spread for EM.

## RESULTS

It should first be noted that the method of preparation of Ad2 DNA (14) involved treatment with Pronase so that the protein tightly attached to the ends of the DNA in the virion was mostly removed. Secondly, in the studies aimed at measuring the length of the inverted terminal repetition, (sections i, ii, and iii below) denatured full-length single strands were examined in the electron microscope. Under the conditions of incubation and spreading, about 50% of the single strands were circular and 50% were linear. For the experiments in sections i and ii, on mapping restriction fragments and a secondary structure feature relative to the ends, the linear strands were studied. For the experiments in section iii on visualizing the inverted terminal repetition, circular single strands were studied.

**(i) Mapping two restriction fragments by**

EM. For the experiments described below, we wished to have as reference regions, a duplex segment close to, but not at an end of, single-stranded Ad2 DNA and a duplex segment at the end. By restriction mapping, the *Eco*RI-E fragment maps from 83.4 to 89.7 U, and the *Hpa*I-E fragment maps from 0 to 4.4 U (12).

Each of these restriction fragments was separately hybridized to denatured Ad2 DNA as described above and mounted for EM by the gene 32-EtdBr technique (21). Of 100 single-strand full-length Ad2 DNA molecules, 52 contained a duplex segment corresponding to the hybridization of the *Hpa*I-E fragment. It was located at the terminal position, and it had a measured length of 4.8 ± 0.4 Ad2 U. Mapping data are shown in Fig. 2, and an electron micrograph is shown in Fig. 3a. This terminal duplex region was also discernible in low formamide-cytochrome *c* spreads (Fig. 3b).

The duplex region of the *Eco*RI-E fragment was observed in 45 out of 100 molecules (Fig. 3c). It was measured as 7.0 ± 0.7 U in length with its closer terminus 11.0 ± 1.4 U from one end (Fig. 2). The EM mapping data are in reasonable agreement with the restriction mapping data (12, 15).

**(ii) Identification of a conspicuous secondary structure feature at 73 Ad2 U and of the terminal duplex projection by low formamide-cytochrome *c* spreading.** Ad2 DNA was denatured and incubated under the conditions for circularization described above. A portion of the incubation mixture was then diluted 10-fold into the low formamide-spreading solution and prepared for EM. Under these conditions, 48 out of 100 full-length single-stranded molecules chosen at random were circles. A prominent secondary structure feature was observed quite frequently on both linear and circular molecules. A total of 72% of all the linear molecules in the sample had this structure. It mapped reproducibly at 27 U from the closer end; thus, the feature is either at 27 or 73 U (Fig. 4a). The contour length of the feature was measured as 480 ± 50 nucleotides, and it was bigger and more conspicuous than any other secondary structure features occasionally observed under this spreading condition (Fig. 4b). An electron micrograph is shown in Fig. 3b.

Denatured Ad2 DNA was hybridized with the *Hpa*I-E fragment and spread, by both cytochrome *c* and gene 32-Etd Br techniques. Since the secondary structure feature was closer to the end opposite the *Hpa*I-E duplex region (Fig. 3a and b), it was assigned the map position of 73 U. This secondary structure feature was then used as a marker to assist in the detection of a terminal duplex projection in circular molecules. All circles with the 73% secondary structure feature were photographed. The shorter distance between all detectable projections and this feature were measured and plotted in Fig. 5a. There was a predominant peak at 27 U. Since there were no secondary structures observed at this distance from the 73% marker in linear molecules (Fig. 4a), we conclude that this projection was a duplex region due to association of the inverted terminal repeats. The measured duplex length was 125 ± 15 ntp (Fig. 5b). A micrograph is shown in Fig. 6a.

**(iii) Visualization of the terminal duplex projection by the gene 32-EtdBr technique.** The 73% secondary structure feature was again detected in 50% of both linear and circular molecules mounted by the gene 32-EtdBr technique; however, its appearance was considerably different. In low formamide basic protein film spreads, it was usually observed as a long hairpin-like structure as in Fig. 3b. By the gene 32-EtdBr technique, it was a small single-strand loop of ca. 500 nucleotides, connected to the rest of the single-strand DNA by a short duplex region with an estimated length of 20 ntp (Fig. 3a). Very similar types of structures were seen for the 2.8 to 8.5 F loop and the loops associated with the 16*S* and 23*S* rRNA genes in $\phi 80 d_3 ilv$ (21). We believe that the Ad2 DNA secondary structure feature is a sequence of length 500 nucleotides flanked by a duplex stalk due to short inverted repeat sequences. The short duplex is stable under the gene 32

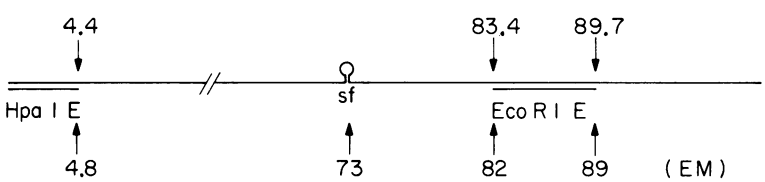



FIG. 2. *Map of the positions of several restriction fragments on Ad2 DNA as determined by gel electrophoresis (upper numbers) (13), and by the present EM study (lower numbers). The position of the conspicuous secondary structure feature is also shown. All coordinates are given in percentages of the total length of Ad2 DNA.*

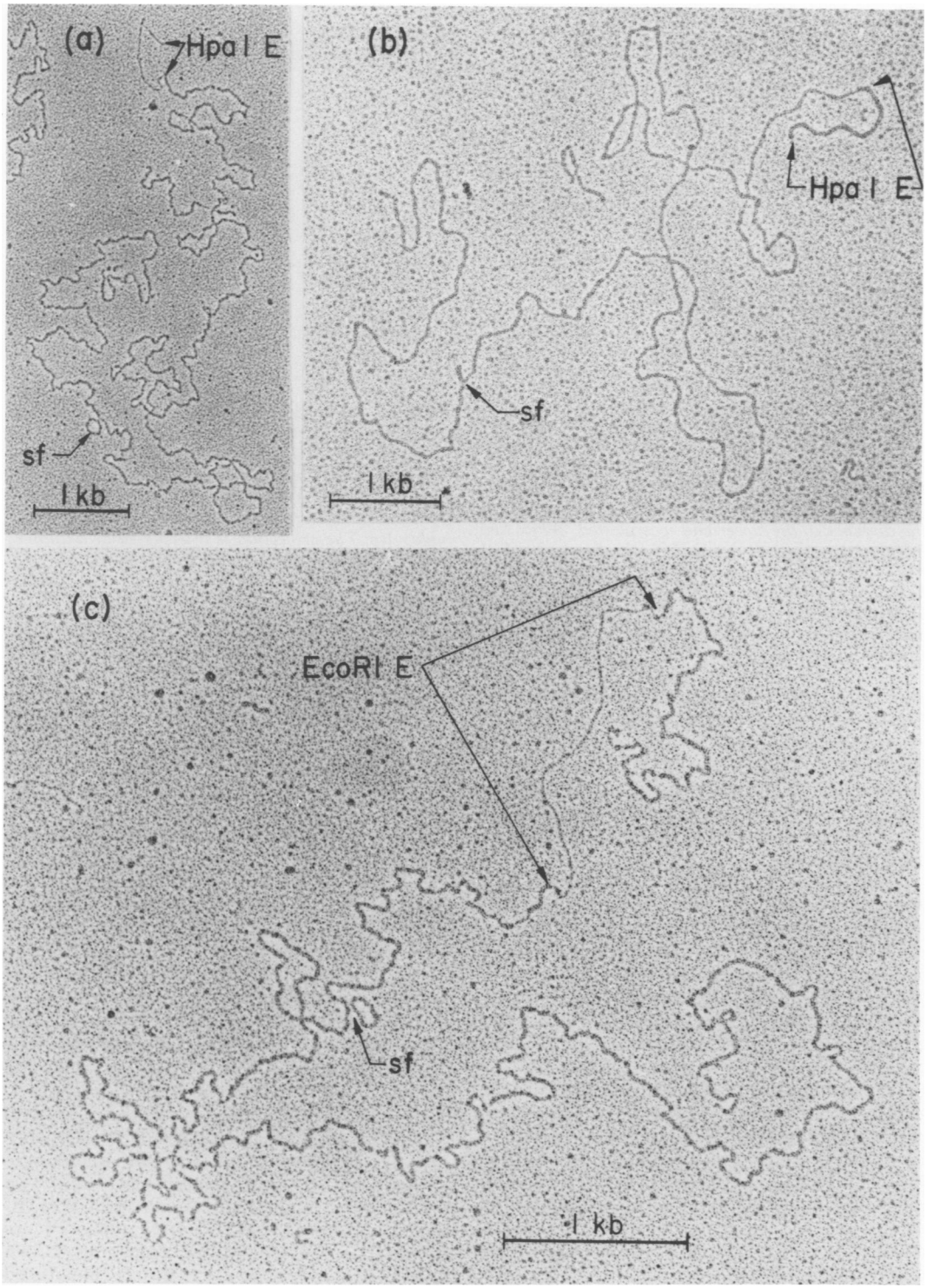



FIG. 3. *Electron micrographs of: (a) a gene 32-EtdBr spread of single-strand Ad2 DNA with a duplex region due to hybridization of the HpaI-E fragment and the secondary structure feature (sf) at 73%; (b) a low formamide-cytochrome c spreading of single-strand Ad2 DNA with the same features as above; (c) a gene 32-EtdBr spread of single-strand Ad2 DNA with a duplex region due to hybridization of the EcoRI-E fragment, and the secondary sf at 73%.*

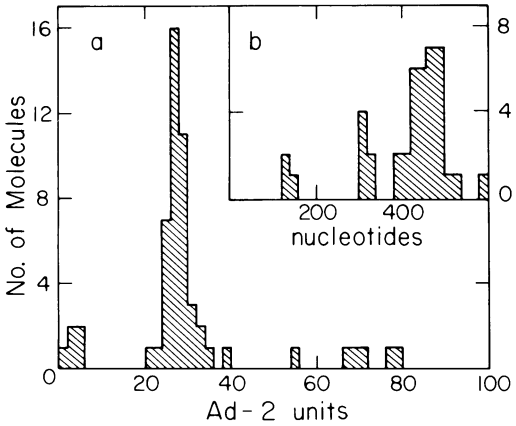



FIG. 4. *(a) Distribution of all secondary features on linear single strands of Ad2 DNA prepared for EM study by low formamide-cytochrome c spreading. On molecules with one secondary structure feature, the distance to the closer end was plotted. When two features were present on one DNA molecule, the feature closer to an end was assigned the smaller coordinate. (b) Distribution of contour lengths of the secondary structure feature located at 27 U from one end. These are single-strand lengths, measured by tracing up and down the feature.*

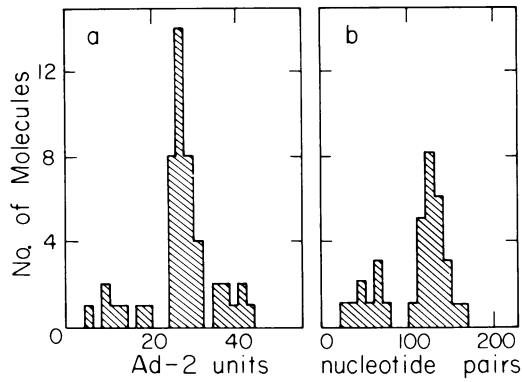



FIG. 5. *(a) Distance between all detectable projections and the 73% secondary structure feature on single-strand circular molecules of Ad2 DNA. Only the closer coordinate is plotted. (b) Length distribution of projections that were located at 27 U apart from the 73% secondary structure feature on circular molecules. The projection is interpreted as a duplex, and the length is given in nucleotide pairs.*

mounting conditions. The appearance of the feature in the low formamide-cytochrome *c* spreads suggests that there are additional pairing regions within the sequence. These may be because they are A+T rich and unstable under the gene 32 incubation conditions that are known to cause selective denaturation of A+T rich regions (6).

The 73% loop could be used as the sole marker to locate the terminal duplex projection (Fig. 6b) in gene 32-EtdBr technique. However, since both the loop and the terminal duplex projection are rather obscure, the *Eco*RI-E fragment was hybridized to the Ad2 single strands as an additional marker for identifying the terminal duplex. In 14 linear molecules, a duplex region of 7.0 ± 0.9 U in length located at 8.3 ± 0.9 U from the 73% loop and 11 ± 1.2 U from the right end were observed (Fig. 3c). These data confirm the mapping data in Fig. 2. A total of twenty-two single-strand circles containing both the 73% loop and the *Eco*RI-E duplex region were photographed. Fourteen of these molecules had a clear short duplex projection located at 12 ± 0.9 U from the closer end of the duplex region and 26.5 ± 1.7 U from the 73% loop (Fig. 6c and d). The length of the short duplex region was measured as 115 ± 10 ntp, in close agreement with the value obtained from the low formamide-cytochrome *c* spreads.

**(iv) Secondary structure features within the terminal inverted repeat region.** A small thick region was often noticed at the end of single-strand Ad2 DNA in low formamide basic protein film spreads. No such thick regions were observed at either end of a single strand of the *Eco*RI-E fragment. These observations suggest that there is some secondary structure close to the terminus. Several systems that permit a more decisive study of the problem were investigated.

The single-stranded ends of Ad2 DNA were exposed by digestion of the duplex DNA with *E. coli* Exo III for 10 min as described above. These molecules and the denatured *Hpa*I-E fragment, both of which contain the inverted terminal repetition, and the *Hin*dIII-B fragment (17.0 to 31.5 U on Ad2 DNA), which does not possess the inverted terminal repetition, were prepared for EM by low-formamide (38%) and regular-formamide (50%) cytochrome *c* spreadings. The number of thick ends in each sample was scored. These data are presented in Table 1. Figure 7a, b, and c show the predominant end structures observed in Ad2: Exo III 10-min samples, single strands of *Hpa*I-E, and single strands of *Hin*dIII-B, respectively. Fifty two percent of all single strand ends in the Exo III-treated Ad2 DNA and 23% of all ends in denatured *Hpa*I-E DNA had thickened ends in 38% formamide, whereas only 4% of denatured *Hin*dIII-B ends showed some thickening. The thick end suggests the existence of a foldback type of secondary structure at the end of single-strand Ad2 DNA. This structure was less frequently observed in 50% formamide spreadings, that is, in more denaturing conditions.

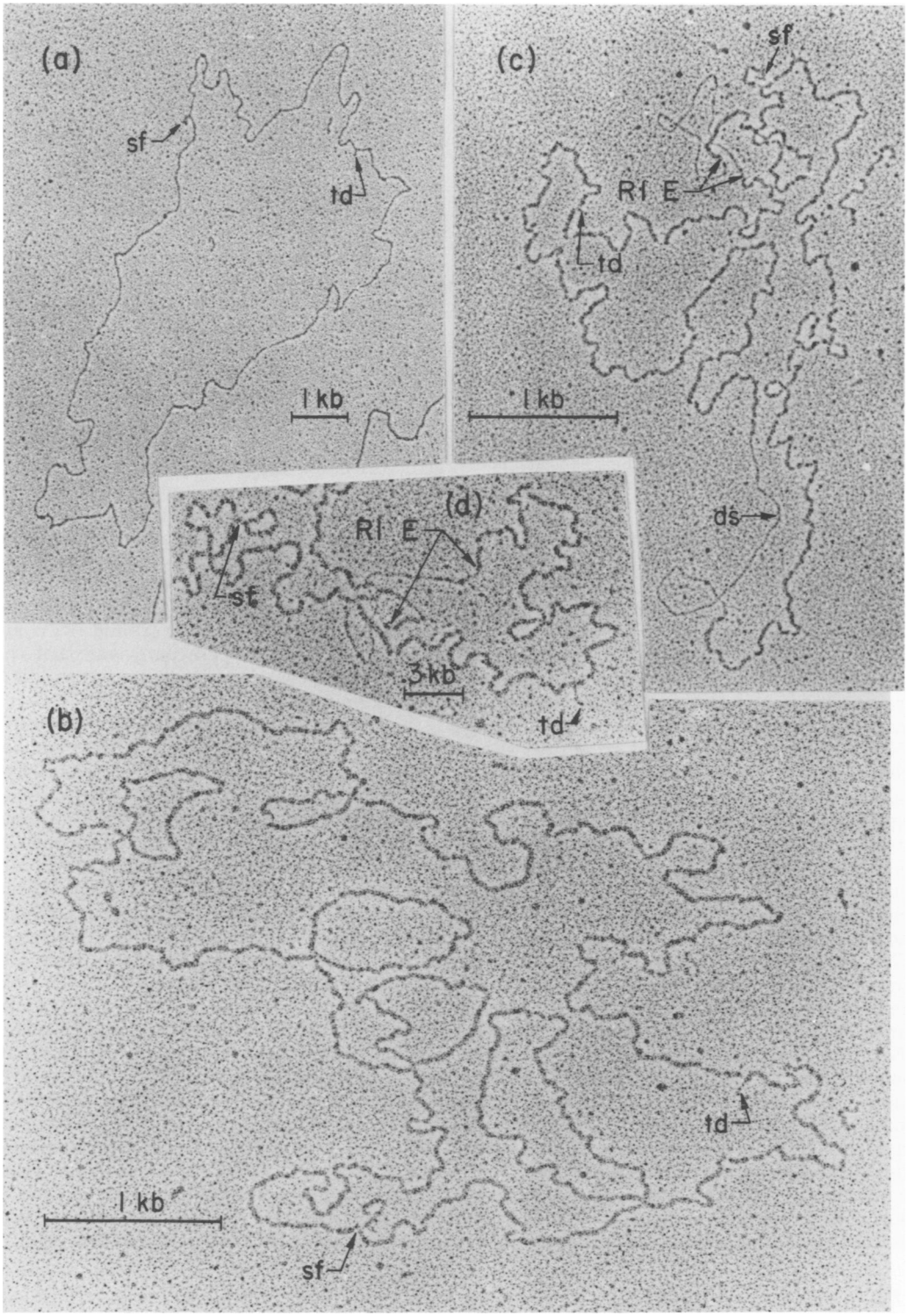



FIG. 6. *Electron micrographs of: (a) a single strand circular molecule prepared by low formamide-cytochrome c spreading. The 73% secondary structure feature (sf) and the terminal duplex projection (td) are identified by arrows; (b) a gene 32-EtdBr spreading of single-strand circular Ad2 DNA containing the 73% secondary structure (sf) and the terminal duplex projection (td); (c) a gene 32-EtdBr spreading of single-strand circular Ad2 DNA with the 73% secondary structure (sf), a duplex segment due to reassociation with the EcoRI-E fragment and the terminal duplex projection (td). This molecule also contains a double-stranded segment (ds) due to reassociation of a short fragment produced by random breakage with the complete single strand; (d) part of a complete single-strand circle of Ad2 DNA showing the 73% secondary structure (sf), the EcoRI-E duplex region and the terminal duplex projection (td) (gene 32-EtdBr method).*

TABLE 1. *Frequency of thick ends in cytochrome c-formamide spreads*[a]

| Spreading condition (%) Formamide | Frequency (%) of thick ends in: | | |
|---|---|---|---|
| | Exo III-treated Ad2 | Denatured *Hind*III-B | Denatured *Hpa*I-E |
| 50 | 20 | 12 | 8 |
| 38 | 52 | 8 | 23 |

[a] About 100 ends in each sample were scored. In each experiment, a mixture of the three kinds of DNA was spread on the same grid. The *Hind*III-B single strands and the *Hpa*I-E single strands have quite different lengths (14.5 and 4.8 U in length, respectively) and are readily distinguished. This procedure controls for variations in focus and in graininess on different grids. Under these circumstances, the differences between thick ends and normal ends are quite apparent to the observer.

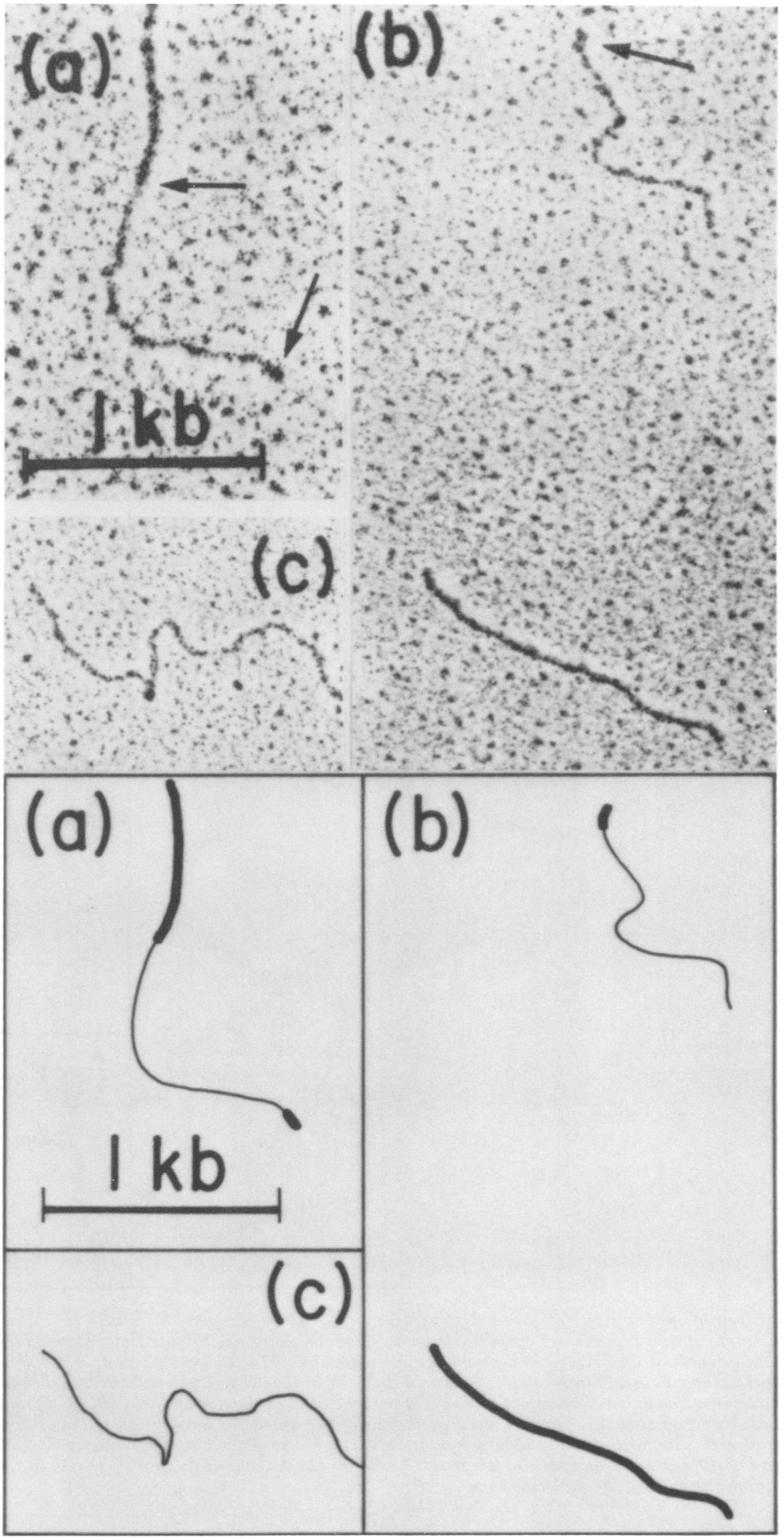
These DNA samples and two other controls, randomly sheared $\phi$X174 DNA and denatured *Eco*RI-E fragments, were prepared for EM study by the gene 32-EtdBr technique. The single-strand ends of Exo III-treated Ad2 DNA as well as those of denatured *Hpa*I-E often displayed unusual structures, whereas a normal straight end (as shown in Fig. 8f) was most frequently observed on the other single-strand DNA molecules. The unusual structures were grouped into 5 classes: (i) an end with a short double-strand projection ending in a thickened region (Fig. 8a); (ii) an end with a short double-strand projection (Fig. 8b); (iii) an end with a small single-strand loop (Fig. 8c); (iv) an end with a terminal bush (Fig. 8d); and (v) a Y-shaped end, with one single-strand branch and one double-strand branch (Fig. 8e).

The frequencies of appearance of the several kinds of structures for various kinds of DNA samples in gene 32-EtdBr spreads were determined as follows. Exo III-treated Ad2 duplex DNA was mounted on the same grids with sheared linear $\phi$X174 single strands (average length, ca. 1.5 kb). Denatured *Hpa*I-E fragments (4.8 U in length) and denatured *Eco*RI-E fragments (7.2 U) were mounted on the same grids. In each case, the experimental DNA was readily distinguished from the control DNA by length. When the ends of different strands were observed side by side, as in the procedure used, the rather considerable variations in background graininess and structure from grid to grid and the effects of variation in focus on appearance were minimized. The differences in appearance between the several kinds of structure described are quite clear on the fluorescent screen of the electron microscope.

The observed frequencies for the several structures are reported in Table 2. Plausible polynucleotide structures corresponding to the observed structures are shown in Fig. 8. In particular, the most frequent classes, a and b, appeared to be foldback structures. A total of 50% of all single-strand ends in Exo III-treated Ad2 DNA and 23% of the ends in denatured *Hpa*I-E fragments showed a complex secondary structure of one kind or another, whereas, only 7 to 8% of the single-strand ends of sheared $\phi$X174 and denatured *Eco*RI-E fragments showed some degree of variation from normal straight ends. The accuracy with which the length of a short region can be measured by EM is probably rather low. We estimate that the short duplex region in types (i) and (ii) is approximately 34 $\pm$ 10 base pairs and that the small terminal single-strand loop in type (iii) is 100 $\pm$ 20 nucleotides in length.

**(v) Gene 32 protein can label the cohesive ends of $\phi$80d$_3$*ilv*.** Since a considerable number of the single-strand ends of Ad2 DNA displayed the structure of type (i), it was of interest to know what is the minimum length of a single-strand stretch that can be detected by gene 32 protein. Duplex DNA of $\phi$80d$_3$*ilv*, has the lambdoid phage type cohesive ends of length 12 nucleotides, T7 DNA which has flush ends, duplex Ad2 *Eco*RI-E fragment, which has staggered ends of 4 nucleotides, and Ad2 DNA were prepared for EM study by the gene 32-EtdBr technique. As reported in Table 3, 43% of the $\phi$80d$_3$*ilv* ends were thickened as though coated with gene 32 protein, but only a small percentage of the ends of the *Eco*RI-E fragment and of T7 DNA showed such thickening. A typical micrograph of a coated end on $\phi$80d$_3$*ilv* is shown in Fig. 8g. Figure 8h shows the clean uncoated end of duplex T7 DNA. To test whether these thickened ends were due to binding of gene 32 or simply to the EtdBr, the DNAs were mounted with EtdBr alone (8, 10). The $\phi$80d$_3$*ilv*

FIG. 7. *Electron micrographs of the end structures prepared by low formamide-cytochrome c spreadings. In this technique, duplex DNA is slightly thicker than single-stranded DNA. The explanatory tracings show the double-stranded and the single-stranded regions. (a) The end of an Ad2 DNA partially digested with E. coli Exo III with an apparent secondary structure feature at the end. An arrow points to this feature; a second arrow points to the junction of duplex DNA with the single-stranded region exposed by Exo III treatment. (b) One double-strand HpaI-E fragment and one single-strand HpaI-E fragment. One end of the single strand fragment is thicker than the other end. The thickening is marked by an arrow. (c) The end of a HindIII-B single-strand fragment. No secondary structure is visible at the end.*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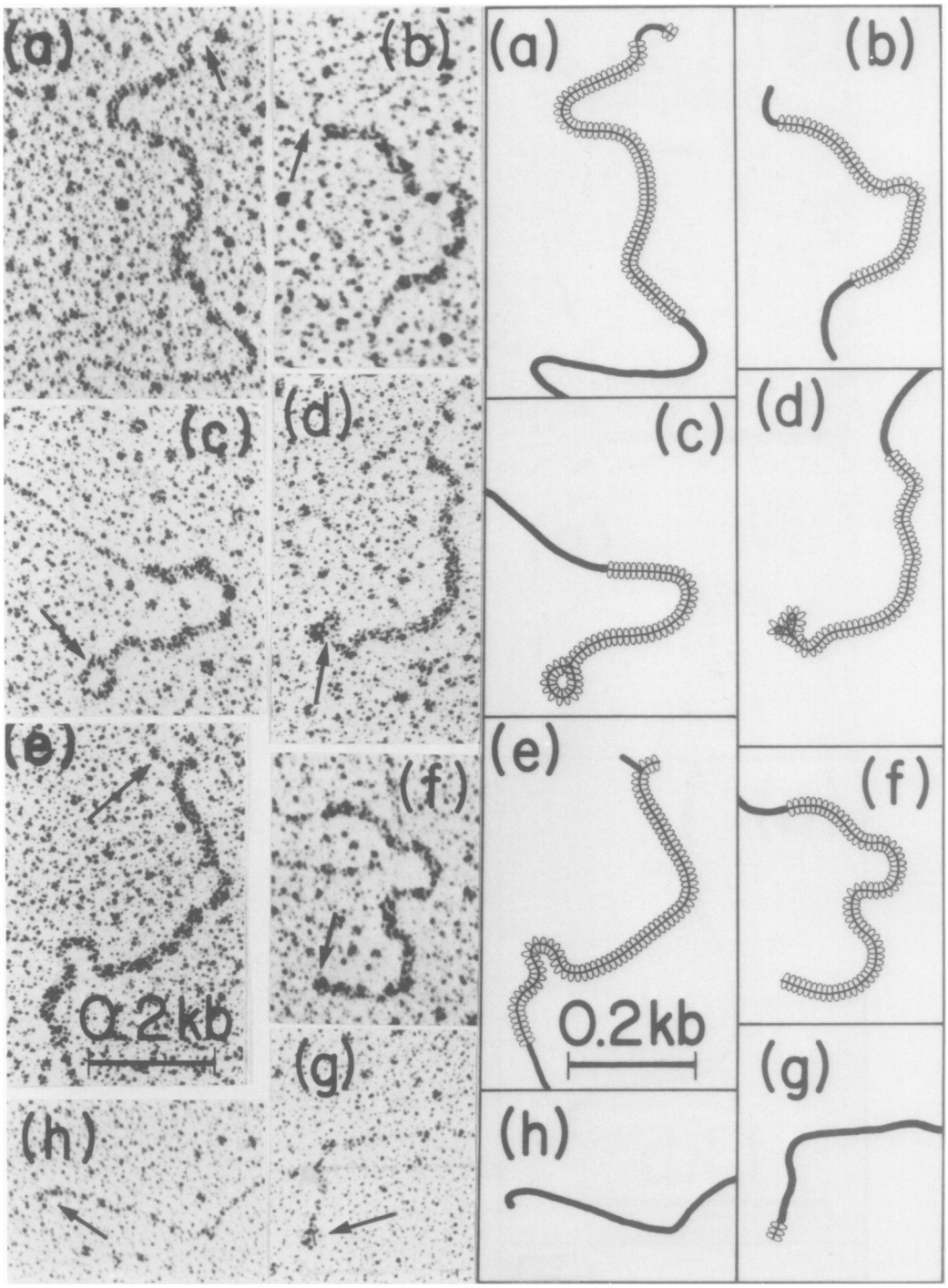


FIG. 8. *Electron micrographs of the end structures prepared by the gene 32-EtdBr technique. In this technique, single strand regions are coated with gene 32 protein and are thicker than duplex regions. The corresponding double-strand region and single-strand region coated with gene 32 protein are shown in the explanatory tracings. Several kinds of end structures observed in Exo III-treated Ad2 DNA as described in the text are shown in a through e. The letters correspond to those used in the text and in Table 2. Arrows point to the terminal secondary structure features. Interpretations of these structures are given in the drawings. (f) A single-strand end without any detectable secondary structure also from Exo III-treated Ad2 DNA. (g) A cohesive end of duplex $\phi 80d_3ilv$ coated with gene 32 protein. (h) An end of duplex T7 DNA. In f, g, and h, arrows point to the ends of the molecules.*

TABLE 2. *Frequencies of different kinds of ends on single-strand Ad2 and control termini, spread by the gene 32-Etd Br technique*[a]

| DNA sample | No. of straight ends[b] | No. of ends with apparent structure | | | | | Total no. of ends with structure[d] |
|---|---|---|---|---|---|---|---|
| | | a[c] | b[c] | c[c] | d[c] | e[c] | |
| Exo III-treated Ad2 | 246 | 118 | 84 | 26 | 19 | 5 | 252 |
| Sheared $\phi$X174 | 100 | 6 | 2 | | 1 | | 9 |
| Denatured *Eco*RI-E Ad2 | 186 | 7 | 5 | | 2 | | 14 |
| Denatured *Hpa*I-E Ad2 | 267 | 41 | 22 | 12 | 4 | 2 | 81 |

[a] For the reasons explained in the legend to Table 1 and in the text, Exo III-treated Ad2 DNA and sheared $\phi$X174 DNA were mixed and mounted on the same grid. Denatured *Eco*RI-E and *Hpa*I-E DNA were mixed and mounted on the same grid.

[b] See Fig. 8f.

[c] See Fig. 8a, b, c, d, and e, respectively.

[d] Of the 498 Exo III-treated ends observed, 252 showed a secondary structure feature. From the $\phi$X174 control, we estimate that 8% of random ends will have an apparent secondary structure feature, so that 41 ± 3% show the specific adeno terminal secondary structure feature. These are all on 5′ ends. The Pronase treatment used for the preparation of the DNA leaves a few amino acids attached to the 5′ ends (Roberts, personal communication) but there are none attached to the 3′ ends. Perhaps these amino acids contribute to the formation of the secondary structure. The fraction of all *Hpa*I-E outside ends with a secondary structure feature may be estimated as follows. Of the 348 *Hpa*I-E ends, 174 are at the *Hp*I-E site and 174 are adeno ends. These are, with equal probability, 5′ ends and 3′ ends. After correcting for the number of secondary structure features in the *Eco*RI-E control fragment, we calculate that 32 ± 4% of the adeno ends have the specific secondary structure feature. These results suggest that there is not much difference between 5′ and 3′ regarding the tendency to form a secondary structure feature. We may positively conclude that secondary structures can form 3′ ends as well as at 5′ ends; however, in view of the small sample studied, we cannot conclude whether or not there is a significant difference in probability of forming the secondary structures between 5′ and 3′ ends.

TABLE 3. *Frequency of clean and coated ends on several duplex DNAs, spread by the gene 32-EtdBr technique*

| DNA sample | No. of clean ends[a] | No. of coated ends[b] | No. of ends with a long single-strand region[c] |
|---|---|---|---|
| T7 | 82 | 4 | 14 |
| $\phi$80d$_3$*ilv* | 50 | 43 | 7 |
| *Eco*RI-E | 96 | 4 | 0 |
| Ad2 | 95 | 3 | 2 |

[a] See Fig. 8h.

[b] See Fig. 8g.

[c] Due presumably to some degradation.

ends did not show the characteristic thickening observed with the gene 32-EtdBr technique.

In summary, these observations show that under the incubation conditions used the 12 nucleotide single-strand cohesive ends of a lambdoid phage DNA are labeled by gene 32 about 43% of the time, whereas the 4 nucleotide ends of the *Eco*RI-E fragment are not.

## DISCUSSION

In the present study the duplex projection from the single strand circle of Ad2 DNA due to the terminal inverted repeat was positively identified. Its length was estimated as 125 ntp by both the low formamide-cytochrome *c* spreading and the gene 32-EtdBr technique. At present, there is no reliable test system for evaluating the accuracy of EM length measurements on segments of this length. However, our measurement is in agreement with the results of Roberts et al. (16) which suggest that the inverted terminal repetition has a length between 100 and 140 base pairs.

The identification of the terminal duplex was greatly assisted by the existence of the secondary structure feature at 73 U in Ad2 DNA. This feature lies within a gene for a protein with a molecular weight of 100,000 and of unknown function (11). The physiological significance, if any, of the secondary structure is unknown.

Before discussing the internal secondary structure within each inverted terminal repeat of Ad2 DNA, we wish to point out the limitations in interpretation of small details of structure observed in nucleic acids by gene 32-EtdBr spreads.

Alberts and Frey (1) estimated that one gene 32 protein binds to 10 to 11 nucleotides of single-strand DNA. A later study (D. E. Jensen, R. C. Kelly, and P. H. von Hippel, Biochemistry, in

press) concluded that: one gene 32 protein binds to 6.7 to 7.5 nucleotides of single-strand DNA; that the association constant of a single gene 32 protein to single-strand DNA is about two orders of magnitude greater than that to duplex DNA; that the binding is cooperative; and that the association constant of gene 32 protein to a DNA site next to a bound protein is estimated as $10^3$-fold greater than to an isolated site.

No specific prediction about the binding to short, single-strand ends can be made from these results, but they are consistent with our observation that a small but observable cluster of gene 32 proteins binds to the 12 nucleotide single-strand ends of $\phi 80 d_3 ilv$ but not to the 4 nucleotide ends exposed by *Eco*RI. Gene 32 protein can denature duplex DNA, and it is conceivable that, on binding to the $\phi 80$ end, a few additional base pairs are broken, permitting more binding of gene 32 protein. Nevertheless, the small size of the thickened spots at the ends of $\phi 80 d_3 ilv$ suggests that only a few protein molecules and 10 to 20 nucleotides are involved. In general then, we tentatively conclude that gene 32 protein can stain a single strand segment of length 10 to 20 nucleotides, but not a shorter one.

Careful observation of the single-strand ends of Ad2 DNA as exposed by Exo III digestion of duplex DNA and in single strand *Hpa*I-E fragments, as compared to single-strand ends of control DNA strands, shows that there is some kind of internal secondary structure within each inverted terminal repeat. The model in Fig. 1 calls for a simple foldback structure. The predominant kinds of end structures seen in the gene 32-EtdBr spreads (Table 2 and Fig. 8) look like foldbacks with (a) and without (b) a loop. The less frequently observed structures (c, d, and e) suggest a more complex secondary structure, perhaps like a tRNA clover leaf. However, we want to emphasize that the resolution of the electron microscope methods used is insufficient to determine the exact nature of the structures observed. For example, clover leaf-like structures with several short single-stranded loops could collapse and look like 8 a or b in electron micrographs. We believe that our observations are strong evidence for some kind of secondary structure, but they do not necessarily support the particular structure illustrated in Fig. 1. We estimate that the average length of the duplex region in either a or b is 34 ± 10 nucleotide pairs. The loop in the structure of type a is quite small, perhaps 10 to 20 nucleotides. Therefore we estimate the number of nucleotides in the secondary structure at the end of Ad2 DNA to be between 50 and 100 nucleotides.

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